Inactivation of Herpes Simplex Virus Types 1 and 2 by Synthetic Histidine Peptides

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Synthetic homologous histidine peptides were found to directly and irreversibly inactivate herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). The inactivation, which occurred within 1 min of virus exposure to the drug, was independent of temperature but dependent upon the pH and molecular size of the polypeptide. Poly-L-histidine consisting of 24 residues (His-24), with a molecular weight (m.w.) of 3,310, inactivated >99% of the virus present at pH 6.0 and 62% at pH 5.0. Poly-L-histidine consisting of 64 (His-64; average m.w., 8,800) or 75 residues (His-75; average m.w., 10,300) inactivated >99% of virus present at pH 5.0 and 6.0. However, His-24, -64, and -75 were not active against these viruses at pH 7.0 or 8.0. At the concentrations tested, poly-L-histidine of 12 (His-12; m.w., 1,665) or 18 (His-18; m.w., 2,487) residues had no effect on HSV-1 or HSV-2 at any of the pHs tested. When these studies were repeated with other basic homologous polypeptides, poly-L-arginine and poly-L-lysine, various degrees of inactivation were observed that were most pronounced in the neutral-to-alkaline pH range. Once virus was inactivated by poly-L-histidine, the effect could not be reversed in vitro by raising the pH to 7.2 or in vivo by injecting the virus into the neutral environment of an animal. These data suggest that the presence of histidine residues in a peptide of suitable structure may endow that peptide with potent antiviral capabilities.

Research on the antiviral effects of cationic polypeptides began appearing in literature more than 30 years ago (2, 8–10, 19). In early studies, investigators examined the effect of different molecular species of poly-L-lysine polypeptides on a variety of viruses. The viruses studied included tobacco mosaic, mumps, Newcastle disease, and influenza, and although inhibitory effects were noted in both tissue culture and animal studies, poly-L-lysine has since been determined to be toxic in mammalian systems (1, 4, 27, 28).

In our laboratory, we have been examining the antifungal properties of synthetic homologous histidine polypeptides (24, 25) because of their structural and functional resemblance to a unique family of naturally occurring histidine-rich polypeptides secreted by the human salivary glands (17, 24, 26). In the course of our investigations into the spectrum of antimicrobial activities of these synthetic histidine peptides, we have observed that poly-L-histidine of appropriate chain length and molecular weight can directly and irreversibly inactivate herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). The specifics of this inactivation in both tissue culture and animal experiments are reported here.

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MATERIALS AND METHODS

Cells and virus. Vero cells were grown and maintained in medium 199 (Flow Laboratories, Inc., McLean, Va.) sup-

plemented with 5% calf serum, 50 µg of gentamicin per ml, and 0.075% NaHCO₃.

Laboratory strains of HSV-1 and HSV-2 were used throughout. The type designation of each virus was authenticated by indirect monoclonal antibody immunofluorescence (31), restriction endonuclease patterns (20), and sensitivity to (E)-5-(2-bromovinyl)-2'-deoxyuridine (5).

Poly-amino acids. Homologous polypeptides of L-histidine containing 12 (His-12), 18 (His-18), and 24 (His-24) amino acid residues with corresponding average molecular weights of 1,665, 2,487, and 3,310, respectively, were synthesized by Peninsula Laboratories (San Carlos, Calif.).

Poly-L-histidine of 64 (His-64) residues and 75 (His-75) residues with average molecular weights of 8,800 and 10,300, respectively, were commercially available from ICN Laboratories (Lisle, Ill.). Stock solutions of poly-L-histidines were prepared at 10 mg/ml in 0.04 M acetic acid and then diluted in medium 199 for use in viral inactivation studies.

Poly-L-lysine (116 residues, 24,300 average molecular weight) and poly-L-arginine (72 residues, 13,900 average molecular weight) were obtained from ICN. These peptides were dissolved in distilled water at a stock concentration of 5 mg/ml. Stock concentrations were further diluted in medium 199 for use in viral inactivation studies.

Animals. All animal studies involved female BALB/c mice of approximately 8 to 10 weeks of age. HSV-1 was incubated with His-75 according to the method described below. Four mice were injected in the soft tissue of the left mandible with the virus-peptide mixture (approximately 2.2 × 10⁵ PFU), four mice were injected with untreated control virus (approximately 2.2 × 10⁵ PFU), and four mice were injected with an equivalent amount of His-75 (5 µg per mouse). The animals were observed daily for signs of viral infection.

Experimental protocol. Unless otherwise stated, the inactivating effect of histidine peptides on virus was investigated as follows. Stock histidine peptides were added to medium

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TABLE 1. Effect of His-75 concentration on inactivation of HSV-1 and HSV-2

Drug concn (μg/ml)	HSV-1 (PFU/ml)		% Reduction[a]	HSV-2 (PFU/ml)		% Reduction
	His-75	Control		His-75	Control	
100	<10	2.8×10^5	>99.99	<10	2.0×10^5	>99.99
10	1.7×10^5	2.8×10^5	39.29	1.7×10^5	2.0×10^5	15.00
1	2.2×10^5	2.8×10^5	21.43	1.2×10^5	2.0×10^5	40.00
0.1	2.9×10^5	2.8×10^5	0.00	2.0×10^5	2.0×10^5	0.0

[a] Calculated by the following equation: % Reduction = 100 − {[treated virus (PFU/ml)/control virus (PFU/ml)] × 100}.

199 to a final concentration of 100 μg/ml, and the pH was adjusted with 0.1 M NaOH to 5, 6, 7, or 8. Control solutions containing an equivalent amount of 0.04 M acetic acid but lacking histidine peptides were prepared in an identical fashion. During early studies, additional controls of medium 199 lacking histidine peptides and 0.04 M acetic acid were included. Approximately 10^5 PFU of virus was mixed with the various histidine peptide solutions. In the standard assay system, virus-peptide mixtures were placed at 37°C for 60 min, and their titers in Vero cells were determined as previously described (6). Modifications of the standard assay system were made to determine the effects of time and temperature of incubation on viral inactivation.

RESULTS

Dose dependence of inactivation of HSV by His-75. The amount of drug required for inactivation of HSV-1 or HSV-2 is shown in Table 1. Greater than 99% of HSV-1 and HSV-2 was inactivated at a His-75 concentration of 100 μg/ml. The percentage of inactivation decreased as the drug concentration decreased. At 10 μg/ml, 39% of HSV-1 and 15% of HSV-2 were inactivated. At 1 μg/ml, 21% of HSV-1 and 40% of HSV-2 were inactivated, while at 0.1 μg/ml, no effect on either virus type was observed. The amount of drug required to inactivate 50% of the virus was calculated by linear regression analysis and found to be 44.36 μg/ml for HSV-1 and 41.15 μg/ml for HSV-2.

pH dependence of inactivation of HSV by His-75. Both HSV-1 and HSV-2 were inactivated at pH 5 and 6 in medium containing His-75 at a concentration of 100 μg/ml (Fig. 1). This inactivation was not observed in His-75-containing medium at pH 7 and 8. Attempts to reverse the inactivation by first exposing the virus to His-75 for 30 min at pH 6 and then rapidly shifting to pH 7.2 for 30 min were not successful. The small amount of inactivation of HSV-2 at pH 8 in Fig. 1 was not reproducible. No appreciable decrease in viral infectivity was noted in controls not exposed to histidine but maintained at the four pHs.

Time and temperature requirements. Studies were continued to establish the conditions required for maximum inactivation of HSV-1 or HSV-2 by His-75. The results of time studies revealed that greater than 99% of HSV-1 or HSV-2 was inactivated within 1 min after exposure to His-75 at 100 μg/ml and pH 6.

Temperature was not critical in the inactivation of HSV by His-75. Inactivation of >99% of HSV-1 or HSV-2 followed a 60-min incubation at 4, 22, or 37°C.

Histidine chain length effect on HSV inactivation. The effect of histidine chain length on the inactivation of HSV-1 was investigated at pH 5, 6, 7, and 8. The smallest molecules tested, His-12 and His-18, had no effect on HSV-1 (Fig. 2). However, His-24 at pH 5 and 6 inactivated 61.9 and >99% of

HSV-1, respectively. The 16% inactivation seen at pH 7 was not reproducible. Both His-64 and His-75 were totally effective in inactivating HSV-1 at pH 5 and 6 but were essentially ineffective at pH 7 and 8.

The effect of His-24 on HSV-2 was also investigated, and the inactivation profile was very similar to that obtained with HSV-1. His-24 was most effective on HSV-2 at pH 6, somewhat effective at pH 5.0, but ineffective at pH 7 and 8 (data not shown).

Interference with inactivation. The possibility that extraneous protein could interfere with His-75 inactivation of HSV was considered. To examine this possibility, calf serum was added to the His-75-inactivating system to final concentrations of 10, 1, or 0.1%. HSV-1 or HSV-2 was then added, and the standard assay was performed. Results presented in Table 2 demonstrate that a 10% serum concentration in the reaction mixture interfered with the efficiency of inactivation
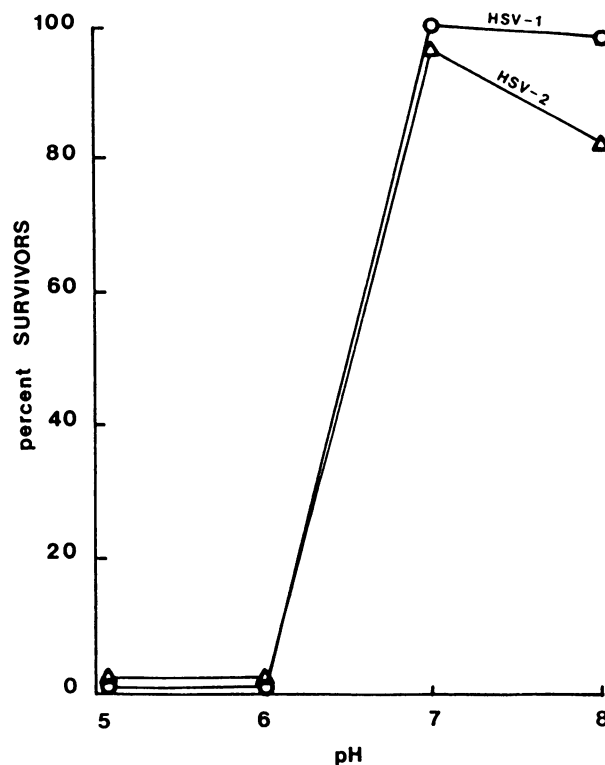

FIG. 1. Inactivation of HSV-1 and HSV-2 by His-75. Approximately 10^5 PFU of either virus was mixed with 100 μg of His-75 per ml in medium 199 at pH 5, 6, 7, or 8 and incubated at 37°C for 1 h. Titers of the surviving virus were determined by the plaque assay and compared with those of control virus which was treated identically but not exposed to His-75.
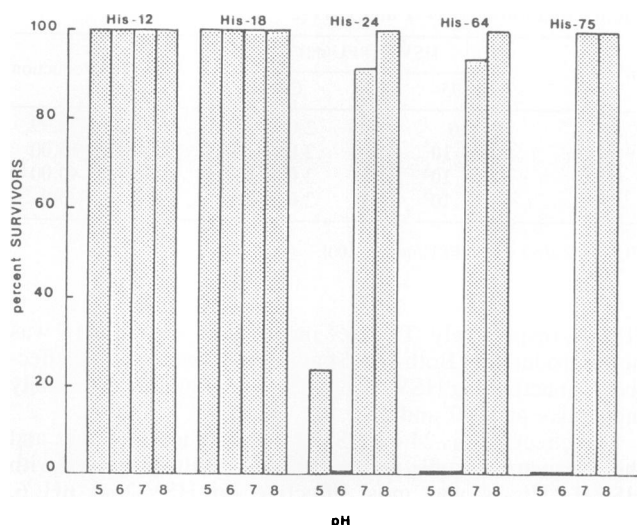
FIG. 2. Inactivation of HSV-1 according to the size of the poly-L-histidine peptide. Approximately 10^5 PFU of HSV-1 was mixed with 100 μg of His-12, His-18, His-24, His-64, or His-75 per ml in medium 199 at pH 5, 6, 7, or 8 and incubated at 37°C for 1 h. Titers of the surviving virus were determined by the plaque assay and compared with those of control virus which was treated identically but not exposed to poly-L-histidine peptides.

of HSV-1 and HSV-2. Only 25% of HSV-1 and 29% of HSV-2 was inactivated. Little or no interference was observed at serum concentrations of 1 and 0.1%, as >99% of both HSV-1 and HSV-2 was inactivated. It should be noted that increasing concentrations of serum in the tissue culture medium resulted in increased precipitation of this polypeptide. Decreased antiviral activity due to serum may, therefore, have resulted from a loss of solubility of the polypeptide, rather than a direct inhibitory effect between peptide and virus.

Animal studies. The four animals injected with His-75-treated virus remained asymptomatic throughout the study. Of the four mice injected with untreated virus, two died (one on day 7 and one on day 9). Both of the surviving mice showed symptoms of disease and nervous system involvement of the cranial nerves. One had severe symptoms including swollen left eye, hair ruffling, lack of mobility, and arched back. The other showed similar but less severe symptoms. The mice injected only with His-75 showed no signs of toxicity.

Poly-L-arginine and poly-L-lysine. The inactivating effects of poly-L-arginine and poly-L-lysine on HSV were examined in the standard assay system. Both peptides were used at 100 μg/ml and exposed to HSV-1 or HSV-2 for 60 min at 37°C. Poly-L-arginine inactivated HSV-1 and HSV-2 at all pHs tested but was generally more active at higher pHs than at

lower pHs (Fig. 3). A similar but less effective pattern was observed for poly-L-lysine inactivation of HSV-1 or HSV-2.

DISCUSSION

The studies presented here demonstrate that poly-L-histidine is a direct and potent inactivator of HSV-1 and HSV-2. This inactivation is independent of temperature but is dependent upon an acidic pH and a chain length of 24 residues or greater and is rapid and irreversible.

A variety of different polypeptides have been described as having anti-HSV activity. Recently it has been reported that arginine- and cystine-rich polypeptides purified from rabbit leukocytes directly inactivate HSV-1 and HSV-2 (16). The antiviral nature of the synthetic histidine polypeptides described in this communication differs in several respects from that of the arginine- and cystine-rich leukocyte polypeptides. Both the histidine and leukocyte peptides directly inactivate HSV-1 and HSV-2; however, the former, in contrast to the latter, inhibit virus infectivity at pH 5 and 6, show no temperature dependence of inhibitory activity, and exhibit apparent irreversible inactivation immediately upon interaction with viral particles.

Poly-L-histidine was a more potent antiviral agent in this pH-dependent assay system than was poly-L-lysine of higher molecular weight or poly-L-arginine of similar molecular weight (Fig. 3). The inability of the histidine peptides to inactivate virus at pH 7 or 8 (Fig. 1), which is in marked contrast to the observed inhibitory effects of poly-L-arginine and poly-L-lysine (Fig. 3), can be explained in part by the differences in the pKs of histidine, lysine, and arginine residues. The imidazole side chain of the histidine residue in poly-L-histidine has been reported to have a pK_a of about 6 (7, 23). This would suggest that, unlike lysine and arginine, the imidazole residues would only be minimally positively charged at pH 7. Therefore, at neutral pH or higher, poly-L-histidine would be unable to bind to the negatively charged surfaces of HSV, which has a reported pK of about 5.1 (29).

One possibility for initial contact between HSV and poly-L-histidine may be the sialic acid residues of viral glycoproteins on the virion surface (13). Notably, poly-L-lysine has been shown to interact with these negatively charged residues on erythrocytes (18). Because the interaction between HSV and poly-L-histidine was irreversible, it seems unlikely that a simple charge-charge interaction alone could account for viral inactivation. If, however, the viral envelope or capsid structure were disrupted or if viral particles were agglutinated by poly-L-histidine, as has been demonstrated for poly-L-lysine aggregation of tobacco mosaic virus (2), then viral infectivity would not be expected to be recovered, even after a shift of pH to a zone unfavorable to poly-L-histidine interaction with HSV. Indeed, if disruption to the structural integrity of the virion were sufficient to expose viral DNA, poly-L-histidine could complex with this nucleic acid, rendering it inactive. Such a hypothesis would

TABLE 2. Effect of serum on His-75 inactivation of HSV

Serum concn (%)	HSV-1 (PFU/ml)		% Reduction[a]	HSV-2 (PFU/ml)		% Reduction
	His-75	Control		His-75	Control	
10	2.7×10^5	3.6×10^5	25.00	2.4×10^5	3.4×10^5	29.41
1	4.0×10^2	2.6×10^5	99.85	2.0×10^3	2.3×10^5	99.13
0.1	<10	2.9×10^5	>99.99	<10	1.8×10^5	>99.99
0	<10	2.0×10^5	>99.99	10	1.4×10^5	>99.99

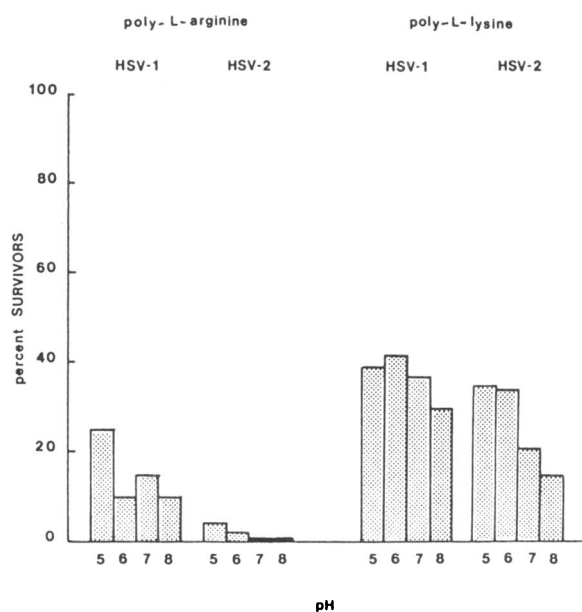[a] Calculated by the following equation: % Reduction = 100 − {[treated virus (PFU/ml)/control virus (PFU/ml)] × 100}.

FIG. 3. Inactivation of HSV-1 and HSV-2 by poly-L-arginine or poly-L-lysine. Approximately 10^5 PFU of HSV-1 or HSV-2 was mixed with 100 μg of poly-L-arginine or poly-L-lysine per ml in medium 199 at pH 5, 6, 7, or 8 and incubated at 37°C for 1 h. Titers of surviving virus were determined by the plaque assay and compared with those of control virus which was treated identically but not exposed to poly-L-arginine or poly-L-lysine.

be consistent with the data of Idoine and co-workers (11), who showed that complexes formed between infectious RNA of equine encephalitis viruses and poly-L-lysine were rendered noninfectious. Infectivity could, however, be recovered from such complexes by degrading the poly-L-lysine with pronase.

The differences noted in the antiviral activity of homologous peptides of various molecular sizes have been observed previously for poly-L-lysine (2, 8). Comparison of the active 24- with the inactive 18-histidine residue peptide suggests that the poly-L-histidine peptide chains may require anchorage to the surface of the virus at additional binding sites, before antiviral activity is observed. Although not concerned with virus studies, other investigators have proposed multiple site interactions to explain the hydrophobic interaction of poly-L-lysine with dipalmitolyl phosphatidylcholine vesicles (3). A similar argument could be invoked to account for the differences noted for the 24-residue, but not for the 64- or 75-residue, peptide, with respect to pH 5 and 6 antiviral activity (Fig. 2). Anchoring at the additional viral site by the 24-residue histidine peptide may be influenced by the localized pH environment, whereas the larger histidine peptides might compensate for this binding site by either different or further extended interactions along the viral surface. An additional explanation for the observed results with the 24-residue peptide may lie in the difference in conformation of the poly-L-histidine molecule at pH 5 and 6. At pH 5, poly-L-histidine is highly protonated and exists in a random coil conformation, whereas at pH 6 (the pK_a of the imidazole group), the peptide is semiprotonated and has α-helical metastable character (21, 22).

Although the majority of the studies presented in this communication have thus far been carried out in tissue culture, the virtually complete inactivation of virus particles

lends optimism to the potential therapeutic effectiveness of poly-L-histidine in vivo. Remarkably, the data show that once inactivated, virus cannot replicate either in vitro in Vero cells or in vivo in mice. Kern and co-workers (15) have noted that a positive relationship exists between the susceptibility of a particular virus in vitro and the efficacy obtained in an animal model infection.

One of the more intriguing aspects of this study is the pH dependence of viral inactivation which occurs at pHs found in regions of the body corresponding to the most common sites of HSV infection. For example, in situ in the mouth, the pH of saliva is approximately 6 (12). In the vagina, a pH range of 3.0 to 6.1 has been reported (14), while the pH of skin has been determined to be in the range of 4.5 to 5.5 (30). Although synthetic histidine peptides were used in this study, it is interesting to speculate that the naturally occurring salivary histidine-rich peptides might also inactivate HSV. Studies are in progress to purify the natural peptides and test their activity against HSV, as well as to examine the inactivating effects of both the natural and synthetic molecules on other viruses.

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